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Infected periodontal granulation tissue contains cells expressing embryonic stem cell markers - a pilot study

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Summary

The commonly practiced removal of granulation tissue during periodontal surgery, aiming to eliminate infection and optimize healing conditions, may also remove progenitor stem cells that could otherwise support periodontal regeneration. The present study aimed to investigate if cells with embryonic stem cell properties are present in periodontal granulation tissue. During the course of flap surgery inflammatory granulation tissue was obtained from four patients and five periodontal defects. Tissues were processed in a collagenase/dispase solution to release the cells. Part of the resulting suspension was processed for bacteriological analysis (IAI PadoTest 4.5), whereas the remaining cell suspension was cultured and passaged once. Upon reaching confluence, total RNA was extracted, followed by cDNA synthesis. PCR was then performed (SYBR Green-based protocols) to measure gene expression levels of Collagen type I, and embryonic stem cell markers Nanog, Oct4, Rex-1 and Sox2. Results are expressed as $2^{-\Delta Ct}$ values of the target gene, calibrated against a housekeeping gene (GAPDH). A high total bacterial load up-to $20.6\pm11.0x10^6$ counts/mg of tissue was found. Collagen type I was strongly expressed, confirming the predominance of mesenchymal/ fibroblastic cells. Among the studied embryonic stem cells markers, Nanog was most highly expressed (2.3±1.2), followed by Oct4 (1.1±0.5), Rex-1 (0.6±0.2) and Sox2 (0.3±0.2). This is the first study that demonstrates the presence of cells expressing embryonic stem cell markers among infected granulation tissue. This knowledge needs to be considered when devising future strategies to improve periodontal wound healing and regeneration.

Zusammenfassung

Parodontitis ist eine entzündliche Erkrankung des Zahnhalteapparates, die mit Weichgewebeverlust und Knochenabbau einhergeht. Die dabei entstehenden Knochendefekte sind mit Granulationsgewebe gefüllt, welches im Zuge von parodontal-chirurgischen Eingriffen entfernt wird. Diese allgemein anerkannte Praktik bezweckt das Schaffen entzündungsfreier Verhältnisse und heilungsfördernder Defektmorphologien. Sie birgt allerdings auch das Risiko der Entfernung von Progenitor-Stammzellen, welche bei Belassen des Gewebes im Defekt eventuell der Regeneration förderlich sein könnten.

Ziel dieser Studie war es, parodontales Granulationsgewebe auf die Anwesenheit von Zellen mit Regenerationspotential, d.h. mit Eigenschaften von embryonalen Stammzellen zu untersuchen. Des Weiteren sollte die bakterielle Besiedlung von parodontalem Granulationsgewebe analysiert werden. Die Studie wurde von der Ethikkomission des Kantons Zürich (KEK-ZH-NR: 2010-0016/0) genehmigt. Alle Patienten wurden über das Ziel und die Durchführung der Studie sowohl mündlich wie auch schriftlich aufgeklärt und haben ihr schriftliches Einverständnis zur Studienteilnahme gegeben.

Das Entzündungsgewebe von fünf Defekten von vier Parodontitispatienten, welche in der Assistentenklinik des Zentrum für Zahnmedizin der Universität Zürich behandelt wurden, wurde entfernt und untersucht. Es handelte sich hierbei um Gewebe aus Resttaschen von mindestens 6 mm Tiefe, welche positiv auf "Blutung auf Sondierung" reagierten. Diese wurden nach erfolgreich abgeschlossener Hygienephase im Rahmen der korrektiven Parodontitistherapie einer routine-mäßigen Lappen-Operation unterzogen. Das Granulationsgewebe, welches im Normalfall verworfen wird, wurde gewogen und danach in einer Kollagenase/Dispase Lösung verarbeitet, um die Zellen aus ihrem Verbund zu lösen. Ein Teil dieser Lösung wurde daraufhin mit einem kommerziell erhältlichen Bakterientest (IAI PadoTest 4.5, Zuchwil, Schweiz) auf den Total Bacterial Load (TBL) untersucht. Die restliche Zellsuspension wurde bei 37 °C und 5 % CO_2 in einem DMEM/F-12 Medium,

welches mit 2 mM L-Glutamin, 1% Penicillin/Streptomycin, 0.05 µg/ml Fungizone (alle Produkte von Sigma-Aldrich, St. Louis, U.S.A.) and 10 % fetalem bovinem Serum (PANSERA ES, PAN Biotech, Aidenbach, Deutschland) versetzt wurde, kultiviert. Nach Erreichen der Konfluenz wurden die Zellen einmal "gesplitted". Es wurde wieder ein konfluierendes Wachstums abgewartet, im Anschluss daran wurde die Total RNA mit dem RNeasy Mini Kit (Quiagen, Hombrechtikon, Schweiz) extrahiert, und einer cDNA Synthese unterzogen. Eine real-time polymerase chain reaction (PCR) wurde mithilfe von SYBR Green-basierten Protokollen durchgeführt, um die Gen Expression von Kollagen Typ I und den embryogenen Stammzell-Markern Nanog, Oct4, Rex-1 and Sox2 zu erhalten. Die Ergebnisse wurden als as $2^{-\Delta Ct}$ Werte des Zielgens, nach Kalibration gegen das "housekeeping Gen" GAPDH, dargestellt.

Bei der bakteriellen Analyse wurde ein hoher Total Bacterial Load (TBL) von bis zu $20.6\pm11.0 \times 10^6$ Bakterien pro mg Gewebe festgestellt. Die Ergebnisse der PCR zeigten, dass Kollagen Typ I stark exprimiert wurde, wodurch die Prädominanz von mesenchymalen/ fibroblastischen Zellen im Granulationsgewebe bestätigt wurde. Unter den untersuchten embryonaler Stammzellmarkern wurde Nanog bei allen Patienten am höchsten exprimiert (2.3±1.2), gefolgt von Oct4 (1.1±0.5), Rex-1 (0.6±0.2) und Sox2 (0.3±0.2).

Diese Studie ist die erste ihrer Art, die Zellen mit Eigenschaften embryonaler Stammzellen im parodontalen Granulationsgewebe nachweist. Diese Erkenntnis sollte bei der Entwicklung zukünftiger Strategien zur Verbesserung der parodontalen Heilung und Regeneration im Auge behalten werden.

Résumé

La parodontite est une maladie inflammatoire des gencives, qui est associée à la perte des tissus mous et une perte osseuse. Les défauts osseux résultant sont remplis avec du tissu de granulation, qui est éliminé au cours de la chirurgie parodontale. Cette pratique a le but de créer conditions sans signes d'inflammation des défauts et morphologies qui encouragent une guérison. Elle porte en elle aussi le risque de l'extraction de cellules souches et progénitrices, qui pourraient éventuellement permettre une certaine régénération.

Le but de cette étude était d'analyser le tissu de granulation parodontale dans la présence de cellules avec un potentiel de régénération, c'est à dire pour examiner les propriétés des cellules souches embryonnaires. En même temps, on voulair analyser la colonisation bactérienne du tissu de granulation parodontale. L'étude a été approuvée par le comité d'éthique du canton de Zurich (ZH-CEC NO: 2010-0016/0). Tous les patients ont été informés de l'objet et la conduite de l'étude à la fois oralement et par écrit et ont donné leur consentement écrit de participation à l'étude.

Dans cette étude pilote, les tissus inflammatoires de cinq patients atteints de parodontite on été retirés et examinés. C'est autour de ce tissu de poches résiduelles d'au moins 6 mm de profondeur, qui a répondu positivement à "saignement au sondage." Ils ont été soumis à la réussite de l'hygiène dans la phase corrective de la chirurgie parodontale. Le tissu de granulation, qui est normalement mis au rebut a été pesé et ensuite traitées dans une solution de collagénase / dispase à dissoudre les cellules. Une partie de cette solution a ensuite été examiné en utilisant un test de bactéries disponibles dans le commerce (IAI PadoTest 4.5, Zuchwil, Suisse) sur la charge bactérienne totale (TBL). La suspension de cellules restantes a été incubées et cultivé à 37 ° C et 5% de CO2 dans un milieu contenant 2 mM DMEM/F-12 L-glutamine, 1% de pénicilline / streptomycine, 0,05 microgrammes / ml Fungizone (tous les produits de Sigma-Aldrich, Saint- Louis, Etats-Unis) et 10% sérum de veau fœtal (ES Pansera, PAN Biotech, Aidenbach, l'Allemagne a été après). Après avoir atteint une

confluence des cellules ont été une fois "panachés". Une nouvelle croissance confluente a été de nouveau attendu, suite à laquelle l'ARN total a été isolé en utilisant le kit RNeasy Mini (Qiagen, Hombrechtikon, Suisse), lavé et soumis à la synthèse d'ADNc. Une réaction en temps réel en chaîne par polymérase (PCR) en utilisant SYBR Green protocoles basés ont été effectuées afin d'obtenir l'expression du gène du collagène de type I et les cellules souches embryonnaires avec les marqueurs Nanog, Oct4, Rex-1 et Sox2. Les résultats sont exprimés comme le 2-ΔCt valeurs comme le gène cible, après l'étalonnage contre le «gène de ménage" GAPDH.

Une relativement grande charge bactérienne (TBL) a été trouvé de 20,6 ± 11,0 x 106 bactéries par mg de tissu. Les résultats de la PCR ont montré que le collagène de type I a été fortement exprimée, ce qui a été confirmé par la prédominance des cellules mésenchymateuses / fibroblastique dans les tissus de granulation. Parmi les tests marqueurs des cellules souches embryonnaires Nanog chez tous les patients a été exprimée au plus haut niveau (2,3 ± 1,2), suivie par Oct4 (1,1 ± 0,5), Rex-1 (0,6 ± 0,2) et Sox2 (0,3 ± 0,2).

Cette étude est la première du genre, qui prouve l'existence des cellules avec les propriétés souches embryonnaires dans les tissus de granulation parodontale. Cette constatation doit être intégrée dans des futures développements pour améliorer au moins la cicatrisation ou même pour favoriser une régénération parodontale.

Introduction

Removal of pocket granulation tissue during periodontal flap surgery procedures was developed with the aim to improve the conditions for wound healing and new attachment formation. Nevertheless, later studies indicated that removal of this tissue in conjunction with flap surgery is not critical for establishing conductive conditions for the efficient healing of the periodontal tissues (LINDHE & NYMAN 1985). Hence, the implications of the removal of this granulation tissue on the repair and/or regeneration process of the periodontium have been interpreted in different ways over time. The concept that progenitor stem cells may reside in the periodontal tissues that give rise to virtually all periodontal tissues was first proposed in the '80s (MELCHER 1985). Studies of the last decade have indeed identified stem cells in the periodontal ligament tissue, with the multi-potent capacity to generate osteoblast-like and cementoblast-like cells *in vitro*, as well as cementum-like and periodontal ligament-like tissues when transplanted *in vivo* in mice (SEO et al. 2004, GRONTHOS et al. 2006).

The fact that granulation tissues formed after tooth extraction can differentiate into bone, filling up the empty socket, indicates that some precursor cells are present (STEINER et al. 2008, TROMBELLI et al. 2008). However, the presence of multi-potent progenitors stem cells has not been demonstrated in the case of infected granulation tissues from chronic periodontitis lesions. It is hypothesized that the commonly practiced removal of granulation tissue during periodontal surgery may also result in removal of vital cells with characteristics of multi-potent stem cells, which could support tissue healing. Better characterization of the nature of the removed granulation tissue may improve our understanding of the conditions that favour regeneration, rather than repair, of the periodontal tissues. Therefore the aim of the present study was to establish primary cell cultures from periodontal granulation tissues removed during the course of routine periodontal surgery, and to investigate the presence and expression levels of characteristic embryonic stem cell markers within these cultures.

Materials and Methods

Patient demographics and diagnosis

Ethics approval for this study was obtained from the Ethics Commission of the Canton of Zürich (KEK-ZH-NR: 2010-0016/0). All patients were treated in the Periodontology postgraduate clinic of the Center of Dental Medicine and have given their informed consent to participate in the study according to the recommendations of the declaration of Helsinki. Periodontal disease was classified as described by the International Workshop for a Classification of Periodontal Diseases and Conditions in 1999 (ARMITAGE 1999). Patients with either severe chronic (CP) or severe aggressive periodontitis (AgP) were included. All patients were non-smokers. They had already received a non-surgical periodontal therapy, and only the first patient had received antibiotics in the course thereof (amoxicillin 500 mg and metronidazole 500 mg 3 times per day for 10 days). Since patients were in need of further treatment, i.e. periodontal surgery in residual pockets of at least 6 mm probing depth that were positive for bleeding on probing, these residual pockets underwent routine periodontal surgery, in the course of which granulation tissue was obtained.

Tissue collection and processing

The removed tissues were first weighted on a precision scale. Thereafter they were processed for 2 h in 1 ml of digestion solution consisting of 3 mg/ml collagenase and 4 mg/ml dispase (Sigma-Aldrich, St. Louis, U.S.A.) in continuous rocking action on a heating block set at 37 °C. One hundret µl of the resulting suspension was used for bacteriological analysis (IAI PadoTest 4.5, Zuchwil, Switzerland), whereas the remaining cell suspension was seeded on 75 mm^2 culture flasks, and cultured further in 5 % CO_2 at 37 °C in DMEM/F-12 media, which was supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, 0.05 µg/ml fungizone (all from Sigma-Aldrich) and 10 % foetal bovine serum (PANSERA ES, PAN Biotech, Aidenbach, Germany). The established cell cultures were passaged once, using Stem

Proacutase Cell Dissociation Reagent (Invitrogen, Paisley, U.K.) during the detachment procedure. Upon reaching confluence, the cell cultures were processed for gene expression analysis.

RNA extraction and cDNA synthesis

Total RNA was extracted from the cells with the RNeasy Mini Kit (QIAGEN, Hombrechtikon, Switzerland), according to the manufacturer's instructions. One μg of extracted total RNA was then reverse transcribed into single stranded cDNA synthesis using M-MLV Reverse Transcriptase, Oligo$(dT)_{15}$ Primers, and PCR Nucleotide Mix according to the manufacturer's instructions (Promega, Madison, U.S.A.), at 40°C for 60 min, and 70°C for 15 min.

Quantitative real-time Polymerase Chain Reaction (qPCR)

For gene expression analysis in the established cell cultures, qPCR was performed using SYBR-Green-based protocols in an ABI Prism 7000 Sequence Detection System and software (Applied Biosystems, Carlsbad, U.S.A.). The studied genes were collagen type I, and the embryonic stem cell markers Nanog, Oct4, Rex-1 and Sox2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control (housekeeping gene). For the amplification reactions, the qPCR SYBR Master Mix was used (Applied Biosystems, Carlsbad, U.S.A.), in combination with oligonucleotide primers, specifically designed for the indicated genes. The oligonucleotide primer sequences are provided in Table I. The standard PCR conditions were 10 min at 95 ºC, followed by 40 cycles at 95 ºC for 15 seconds, 60 ºC for 1 min, and 72 °C for 30 seconds. The expression levels of these five target genes in each sample were calculated by the comparative Ct method ($2^{-\Delta Ct}$ formula), after being normalized to the Ct value of the GAPDH housekeeping gene.

Microbiological analysis

Part of the digested tissue suspension was immediately sent at the Institut für angewandte Immunologie (IAI) AG, Zuchwil, Switzerland for microbiological analysis using a commercially available microbiological test (IAI Pado Test 4.5). This RNA-based molecular method enables the quantification of bacterial ribosomal 16s rRNA, providing a reliable numeric estimate for total or specific viable bacterial cells in the samples.

Results

Patient and periodontal site defect characteristics are provided in Table II. The five tissue samples were attained from infrabony defects of four patients. One of the patients provided two samples, originating from an infrabony as well as from a furcation defect. Upon surgical removal, each of the five samples was weighted. On average, tissue weight was 53 ± 39.3 mg. After dispersion of the tissue, part of the suspension was forwarded for microbiological analysis by the IAI Pado Test 4.5 (Table II). A high total bacterial load was evident, which was on average 26.4 ± 11.3 x 10^6 counts/ mg of tissue. The presence of specific putative periodontal pathogens was also investigated. *Aggregatibacter actinomycetemcomitans* was detected in 0/5 granulation tissues, *Porphyromonas gingivalis* in 2/5, *Tannerella forsythia* in 1/5 and *Treponema denticola* in 2/5. The cell cultures established from these granulation tissues were analyzed by qPCR for the expression of various genes. Among them, collagen type I was expressed at high levels by the cells, at a relative expression of 144.1 ± 46.7. The expression of four consensus embryonic stem cell markers, namely Nanog, Oct, Rex-1 and Sox2, was further analyzed (Fig. 1). It was found that these markers were indeed expressed by the cells in culture, but at levels 2-log lower than Collagen type I. Among these four studied genes, Nanog was the most highly expressed (2.3 ± 1.2), followed by Oct4 (1.1 ± 0.5), Rex-1 (0.6 ± 0.2) and Sox2 (0.3 ± 0.2).

Discussion

Earlier studies have identified the presence of cells with characteristics of putative mesenchymal stem cells in regenerating periodontal tissues. Such cells were localized particularly in the paravascular and extravascular regions of non-infected healing granulation tissue, based on the detection of the markers STRO-1, CD44 and CD146 (LIN et al. 2008). In a recent study, stem cells with osteogenic, cementogenic and adipogenic capacities were also isolated from inflamed human periodontal ligament tissue, demonstrating that the periodontal ligament tissue can retain its regenerative potential even under inflammatory conditions (PARK et al. 2011).

The present study was designed to investigate the expression of embryonic stem cell markers in cell cultures established from gingival granulation tissues removed during the course of periodontal flap surgery. The high total bacterial load detected confirms the presence of infection in these tissues. This observation is not surprising as it could be expected that granulation tissue next to a periodontal defect would not be sterile, and that non-surgical therapy may not completely eliminate the bacteria. It is possible that these bacteria have either colonized the pocket epithelium or invaded deeper into the periodontal (potentially granulation) tissue. However, it is important to note, that the selected periodontal pathogens were only detected occasionally. On the other hand, the high gene expression of Collagen type I in the established cell cultures confirms that the cell populations contained within the granulation tissue are of mesenchymal or fibroblastic origin. Earlier studies have indeed confirmed the presence of diverse fibroblastic populations within the gingival granulation tissues, which exhibit lower proliferation rates compared to gingival fibroblasts from healthy tissues (LARJAVA et al. 1989, HAKKINEN & LARJAVA 1992). Recent evidence suggests that granulation tissue obtained from intrabony defects during surgery contains mesenchymal stem cell populations that express the STRO-1 marker (HUNG et al. 2011). The present study is the first to confirm the existence of cells with embryonic stem cell properties in periodontal

granulation tissue. To this extent, the cell cultures established from these granulation tissues expressed a panel of embryonic stem markers, including Oct4 (BOIANI et al. 2004), Rex-1 (BEN-SHUSHAN et al. 1998), Nanog (ZHANG et al. 2009, BAIS et al. 2012), and Sox2 (AVILION et al. 2003), which are crucial for the pluripotent capacities of stem cells. All four markers were indeed expressed in the cell cultures obtained from all five granulation tissues. This provides evidence that periodontal granulation tissue contains cell with embryonic stem cell properties. Among these markers, Nanog demonstrated the highest expression, followed by sequentially 2-fold lower levels of Oct4, Rex-1 and Sox2, sequentially. It is not clear at this stage how these markers can contribute to the cell dynamics of the gingival tissue, or what is the importance of the relative expression levels of each one of them. Nevertheless, the present findings imply that surgical removal of granulation tissue inevitably results in removal of pluripotent stem cells that might potentially contributed to the healing of the tissue, once the infection is controlled. This knowledge needs to be considered further in treatment approaches that aim in optimal periodontal wound healing.

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Table I. Oligonucleotide primer sequences (forward and reverse primer)

Gene	Accession Number	Sequence
Collagen type Iα1	(NM_000088)	Forward: AAGATGGACTCAACGGTCTC Reverse: CAGGAAGCTGAAGTCGAAAC
NANOG	(NM_024865)	Forward: CCTTCCAATGTGGAGCAACC Reverse: CTCGCTGATTAGGCTCCAAC
OCT4	(NM_002701)	Forward: GTTGATCCTCGGACCTGGCT Reverse: ATCGGAGTTGCTCTCCACCC
SOX2	(NM_003106)	Forward: AGATGCACAACTCGGAGATC Reverse: GTCATGGAGTTGTACTGCAG
REX1	(NM_020695)	Forward: ATGGCGTCCAAGACTACCAC Reverse: TGAGCGAGAAGCTGGTCTTG
GAPDH	(NM002046)	Forward: CAGCCTCCCGCTTCGCTCTC Reverse: CCAGGCGCCCAATACGACCA

Table II. Patient, site and tissue characteristics

	Patient and site characteristics					**Tissue characteristics**	
	Age (yrs)	**Gender**	**Diagnosis**	**Pocket depth (mm)**	**Defect morphology**	**Tissue weight (mg)**	**TBL/ weight***
Sample 1	41	f	CP	8	Intrabony	25	30.6
Sample 2	18	f	AgP	7	Intrabony	28	43.5
Sample 3	42	f	CP	6	Intrabony	42	18.7
Sample 4	36	m	AgP	8	Furcation	47	24.3
Sample 5	36	m	AgP	14	Intrabony	121	14.9
Average	34.6	N/A	N/A	8.6	N/A	52.6	26.4
SD	9.7	N/A	N/A	3.1	N/A	39.3	11.3

Abbreviations: yrs - years; f - female; m - male; CP - chronic periodontitis; AgP - aggressive periodontitis; TBL - total bacterial load; * TBL x 10^6 counts / mg of tissue.

Figure 1

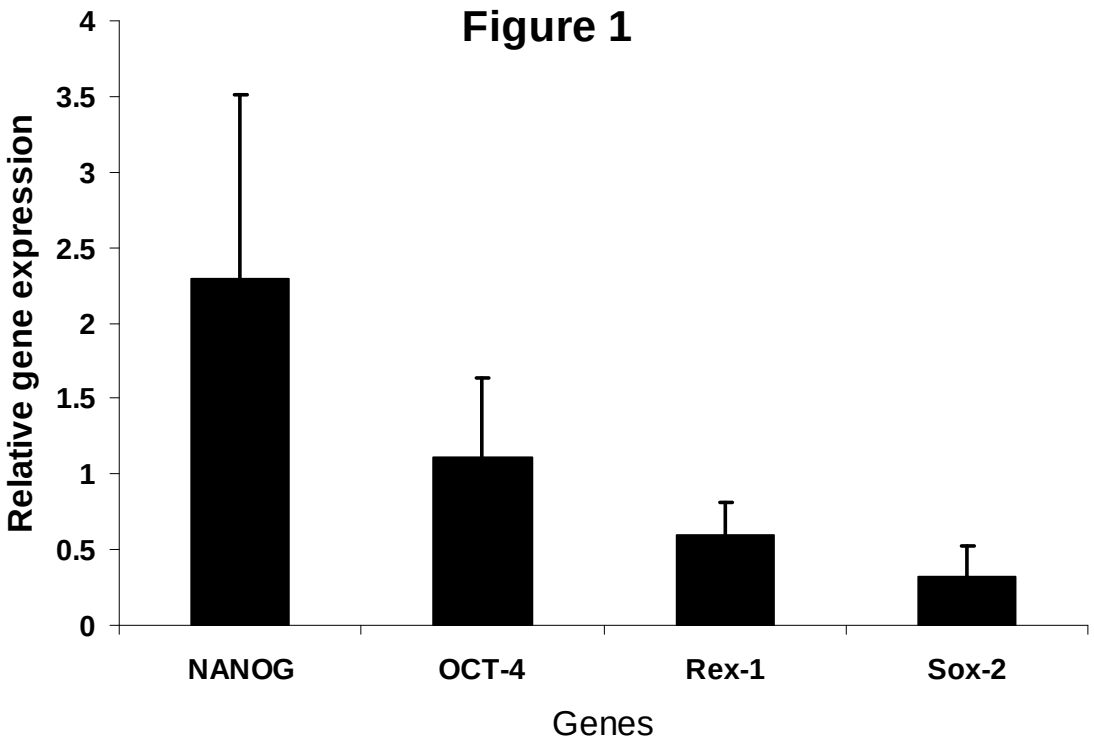

Figure 1. Gene expression of embryogenic stem cell markers in cells cultures established from infected gingival granulation tissues. The relative expression of the genes is expressed as the $2^{-\Delta Ct}$ formula. The bars represent the mean relative expression ± standard deviation of the five cell cultures obtained by the respective granulation tissues.

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